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Phoenixin: a candidate pruritogen in the mouse

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Abstract

Phoenixin (PNX) is a 14-amino acid amidated peptide (PNX-14) or an N-terminal extended 20-residue amidated peptide (PNX-20) recently identified in neural and non-neural tissue. Mass spectrometry analysis identified a major peak corresponding to PNX-14, with negligible PNX-20, in mouse spinal cord extracts. Using a previously characterized antiserum that recognized both PNX-14 and PNX-20, PNX-immunoreactivity (irPNX) was detected in a population of dorsal root ganglion (DRG) cells and in cell processes densely distributed to the superficial layers of the dorsal horn; irPNX cell processes were also detected in the skin. The retrograde tracer, Fluorogold, injected subcutaneously (s.c.) to the back of the cervical and thoracic spinal cord of mice, labeled a population of DRG, some of which were also irPNX. PNX-14 (2, 4 and 8 mg/kg) injected s.c. to the nape of the neck provoked dose-dependent repetitive scratching bouts directed to the back of the neck with the hindpaws. The number of scratching bouts varied from 16–95 in 30 min, commencing within 5 min post-injection and lasted 10–15 min. Pretreatment of mice at –20 min with nalfurafine (20 µg/kg, s.c.), the kappa opioid receptor agonist, significantly reduced the number of bouts induced by PNX-14 (4 mg/kg) compared with that of saline-pretreated mice. Our results suggest that the peptide, PNX-14, serves as one of the endogenous signal molecules transducing itch sensation in the mouse.

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Keywords

dorsal root ganglion; itch; kappa opioid receptor; nalfurafine; primary afferent neuron; pruritogen; pruritus

Introduction

Two novel peptides, phoenixin-14 amide (referred to herein as PNX-14) and phoenixin-20 amide (PNX-20), have recently been isolated and identified in rat brain and spinal cord as well as non-neural tissues including the heart (Lyu et al., 2013; Yosten et al., 2013). PNX-14 is identical among multiple species including human, rat, mouse, porcine and canine; whereas PNX-20 differs in one amino acid between the coding region of human, canine or porcine sequences (Yosten et al., 2013). The precursor of phoenixin is an uncharacterized protein C4orf52 (or *Smim20*), which contains a glycine residue that can undergo C-terminal amidation, and several conserved dibasic residues after glycine, indicative of potential carboxypeptidase cleavage sites (Fricker, 2012). The most abundant peptide generated from C4orf52 is a 14 residue peptide, DVQPPGLKVWSDPF-amide, termed PNX-14. An N-terminal extended peptide, with a sequence of AGIVQEDVQPPGLKVWSDPF-amide, is co-expressed with PNX-14 in tissue samples, such as the heart and hypothalamus, which we called PNX-20 (Yosten et al., 2013).

Insofar as the distribution of PNX-14 or PNX-20 in neural tissue is concerned, immunohistochemical studies show that PNX-immunoreactive (irPNX) cells are expressed in several regions of rat brain including hypothalamus, brainstem and pituitary (Yosten et al., 2013). Because PNX antiserum, a rabbit polyclonal directed against the peptide PNX-14, cross-reacted 100% with PNX-14 amide or PNX-20 amide (Yosten et al., 2013), the term irPNX referred to irPNX-14 and/or irPNX-20 indiscriminately. In the rat spinal cord, irPNX is expressed in networks of nerve fibers distributed to superficial layers of the dorsal horn, and in small-to-medium dorsal root ganglion cells (Lyu et al., 2013). This pattern of distribution of irPNX in the dorsal horn and DRG is similar to that of several sensory peptides including substance P (SP), calcitonin gene-related peptide (CGRP) and gastrin-releasing peptide (GRP). The latter is reported to be an endogenous ligand, acting on gastrin-releasing peptide receptors (GRPR), which promote itch sensation in the mouse (Sun and Chen, 2007). A similar expression pattern of PNX and GRP in the spinal cord and DRGs raised the intriguing possibility that PNX-14 may serve as one of the heretofore unrecognized signaling molecules that transduces itch sensation (Davidson and Giesler, 2010; Sun and Chen, 2007; Yosipovitch et al., 2003).

Methods and Materials

Experimental animals

Male Swiss-Webster mice (20–25 g) were purchased from Taconic Biosciences (Hudson, NY). Animals were housed under a 12 hr light/dark cycle with food and water available *ad libitum*. Experimental procedures were approved by the Temple University Institutional Animal Care and Use Committee, in accordance with the 1996 NIH Guide for the Care and

Use of Laboratory Animals. Experiments were designed to keep the number of animals to a minimum and care was taken to minimize pain or suffering.

Identification of phoenixin from mouse spinal cords

Swiss-Webster mice ($n = 20$) were anesthetized with 4% isofurane and decapitated. Spinal cords, with a total wet weight of 1.6 g, were mixed with 1.6 g of 0.8 mm silica beads (OPS Diagnostics, Lebanon, NJ), divided into 8 polypropylene micro-centrifuge tubes, homogenized in 0.8 ml 5% acetic acid and heated at 95°C for each tube for 5 min, which yielded a total of 4.8 ml of homogenates. After spinning the homogenates at $10,000 \times g$ for 20 min, the supernatant was transferred to a 15 ml polypropylene tube. Commercially available Bicinchoninic Acid (BCA) Protein Assay kit (Life technologies, Grand Island, NY) was used to quantify the protein content in tissue homogenates. To identify phoenixin peptide, 0.8 mg of tissue homogenates were further affinity purified using MagnaBind beads (Life technologies, Grand Island, NY) which had been conjugated to anti-phoenixin antibody. Briefly, saturating amounts of phoenixin immunoreactive peptides from tissue homogenates were bound to antibody conjugated magnetic beads after 4 hr incubation at 4°C; the latter were washed four times with 1 ml of phosphate buffered saline (PBS). After the last wash, beads were transferred to new tubes. The captured peptides were eluted from beads with 50 μ l of 60% acetonitrile in 1% trifluoroacetic acid. Lastly, 3 μ l of the eluent from magnetic beads was directly applied to the MALDI-TOF plate of Maxima LNR (Kratos-Shimadzu Co., Kyoto, Japan), after mixing with 1 μ l matrix solution of alpha-cyano-4-hydroxycinnamic acid for the identification of phoenixin. In the final stage of verification of purified phoenixin, the bioinformatics predicted phoenixin peptide that had been synthesized, and the purified phoenixin were processed by the same procedures as indicated in the previous publications (Lyu et al., 2013; Yosten et al., 2013). A comparable molecular mass on MALDI-TOF and HPLC profiles of the purified peptide and synthetic phoenixin confirmed the molecular identity of phoenixin.

Immunohistochemistry

Animals anesthetized with 4% isofurane were intracardially perfused with PBS followed by 4% paraformaldehyde in PBS. Spinal cords and DRG were dissected, and several pieces of 5×5 mm skin patches were clipped and removed from the back of the mouse, postfixed for 2 hr and stored in 30% sucrose/PBS overnight. Tissues were processed for irPNX by the immunofluorescent method (Lyu et al., 2013).

Tissues were sectioned to 40 μ m thick by a cryostat, blocked with normal goat serum (1:100 dilution in PBS, 0.5% bovine serum albumin, 0.4% Triton X-100), rinsed and then incubated in PNX antiserum (1:1,000 dilution; a rabbit polyclonal against PNX-14; Phoenix Pharmaceuticals Inc., Burlingame, CA). After thorough rinsing, sections were incubated in biotinylated anti-rabbit IgG (1:100 dilution; Vector Laboratories, Burlingame, CA) for 2 hr, rinsed with PBS, and incubated in Texas Red or avidin fluorescein isothiocyanate (FITC, 1:50 dilution) for 5 hr. Sections were washed with PBS, mounted on subbed slides, covered with Citifluor mountant medium (Ted Pella Inc., Redding, CA) and coverslipped.

Fluorogold injection

In four mice, the retrograde fluorescent tracer Fluorogold (3% solution, 10 µl, Biotium Inc., Haywood, CA) was injected s.c. to three spots, separated by 5 mm, to the nape of the neck. Three to five days later, animals under 4% isofurane anesthesia, were intracardially perfused with chilled PBS followed by 4% paraformaldehyde. DRG removed from cervical, thoracic and lumbar segments were processed for irPNX using the fluorescent methods described above. As preliminary results showed that the fluorescence intensity of Fluorogold in DRG sections was less than optimal, an additional step was carried out involving incubation of DRG sections in anti-Fluorogold antibody (Chemicon International, Inc.) followed by incubation in anti-rabbit secondary antiserum conjugated to FITC to amplify the signal. Sections were examined under a confocal scanning laser microscope (Leica TCS SP5, Heidelberg, Germany) with excitation wavelengths set to 488 nm for FITC and 561 nm for Texas Red.

Pre-absorption protocol

To assess cross-reactivity between PNx antiserum and PNx-14 or PNx-20 peptide, spinal cord or DRG sections were incubated overnight with PNx antiserum pre-absorbed with either PNx-14 amide or PNx-20 amide (1 µg/ml). Additional control studies were performed in which spinal cord or DRG sections were incubated overnight with PNx antiserum pre-absorbed with substance P (1 µg/ml), gastrin-releasing peptide (GRP, 1 µg/ml) or calcitonin-gene related peptide (CGRP, 1 µg/ml). Tissues were then processed with the pre-absorbed PNx antiserum in a manner similar to that described for PNx antiserum.

Scratch protocol

The methodology originally introduced by Kuraishi et al. (1995) was employed here and was similar to that described in our earlier studies (Inan et al., 2009; 2011; Zhang et al., 2015). Mice were acclimated individually in rectangular plastic observation boxes for at least 2 hr prior to experiments, which were conducted between 11:00 and 16:00 hr. As the major peak in the mass spectrometry corresponds to PNx-14 in the rat/mouse spinal cord (Lyu et al., 2013), PNx-14 was the primary target for investigation here. PNx-14, dissolved in saline, was injected s.c. to the midline behind the neck, to provoke scratching; an equal volume of saline (0.25 ml/25 g) injected s.c. served as control. The incidence of hindpaw scratching directed to the back of the neck was monitored for 30 min. Animals were euthanized immediately thereafter.

Nalfurafine, the kappa opioid receptor agonist, attenuates scratching in mice associated with several chemically diverse pruritogens (Inan et al., 2009; Zhang et al., 2015). Here, nalfurafine (20 µg/kg in the flank) or saline was injected s.c. 20 min before our standard submaximal dose of PNx-14 (4 mg/kg) and the number of scratching bouts was counted for 30 min.

Chemicals and reagents

PNx antiserum, SP[tyr⁸], GRP, PNx-20 amide peptide, and PNx-14 amide peptide were gifts from Phoenix Pharmaceuticals, Inc., Burlingame, CA. Fluorogold antiserum was purchased from Chemicon International, Inc., Temecula, CA. Nalfurafine was a generous

gift from Adolor Company, Exton, PA. Unless stated otherwise, additional chemicals and reagents were purchased from Sigma Aldrich, St. Louis, MO.

Statistics and data analysis

Data analysis and statistics were performed with GraphPad Prism 6.0 software. The statistical significance of differences between control and experimental groups was calculated by the Kruskal-Wallis one-way analysis of variance followed by Dunn's multiple comparison test. Differences were considered to be statistically significant with * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. All data and histograms are presented as mean with associated standard deviation (SD).

Results

Mass spectrometry detection of phoenixin in mouse spinal cords

Fig. 1 shows a mass spectrometry analysis of chemicals from mouse spinal cord homogenates, where the major peak corresponded to the peptide PNX-14 amide; whereas peaks corresponding to PNX-20 amide were small and not distinct. In addition, a peak corresponding to PNX-17 amide was visible in the spectrometry, suggesting phoenixin may exist in several isoforms with amino acids varying from 14 to 20 in different tissues.

Phoenixin immunoreactivity in mouse spinal cords, dorsal root ganglia, and skin

The PNX antiserum used here has been shown to cross-react 100% with PNX-14 amide and PNX-20 amide (Yosten et al., 2013). For this reason, attempts were not made to distinguish PNX-14 immunoreactivity from PNX-20 immunoreactivity, which is collectively referred to herein as phoenixin immunoreactivity (irPNX).

In the mouse spinal cords ($n=9$), networks of irPNX nerve fibers and/or endings were densely distributed to the superficial dorsal horn of all segments i.e., cervical, thoracic, lumbar and sacral. A cervical spinal section where irPNX cell processes were densely distributed to superficial layers is illustrated in Fig. 2A and B. A few strands of irPNX cell processes extended into deeper laminae, and to an area dorsal to the central canal; irPNX cell bodies were not detected in the dorsal horn or ventral horn (Fig. 2A and B).

With respect to DRG, irPNX of varying intensities was detected in a population of ganglion cells (Fig. 2C). The majority of irPNX neurons had a cell diameter ranging between 20 and 40 μm , similar to that reported for rat DRG (Lyu et al., 2013).

In the skin, irPNX cell processes, which often had the appearance of bead-like structure, were detected in epidermis and dermis of all skin patches examined (Fig. 3A and B)

Fluorogold and PNX-positive DRG

To identify the source of irPNX nerve fibers underneath the skin, the retrograde tracer Fluorogold (3%) solution was injected s.c. to the back of four mice. Three to five days later, DRG from cervical, thoracic and lumbar segments were harvested and DRG sections were then processed for irPNX. In 15 DRG sections, a total of 729 Fluorogold-filled DRG cells

were noted, of which 468 cells were irPNX, and 254 cells expressed both Fluorogold and irPNX. A section of cervical DRG where cells were labeled with Fluorogold and/or irPNX is illustrated in Fig. 4.

Pre-absorption protocol

The specificity of PNX antiserum was assessed with the pre-absorption protocol (Lyu et al., 2013). Spinal cord sections processed with PNX antiserum pre-absorbed with the peptide PNX-14 showed no irPNX in the dorsal horn, as illustrated in Fig. 2D, suggesting nearly 100% cross-reactivity of PNX antiserum with the peptide PNX-14. In contrast, spinal cord sections processed with PNX antiserum pre-absorbed with three other peptides SP, GRP and CGRP, revealed no detectable change of the distribution or intensity of irPNX cell processes in the dorsal horn (not shown), indicating there is no apparent cross-reactivity of PNX antiserum with the three peptides in question.

Phoenixin-14 amide and scratching bouts in mice

As PNX-14 is most abundantly expressed in rat (Lyu et al., 2013) and mouse spinal cords (the present work), PNX-14 was used in the pharmacological studies described below, with the exception of one series of experiments in which the effect of PNX-20 was assessed.

PNX-14 (2, 4, 8, or 16 mg/kg) by s.c. injection to the midline of the neck provoked repetitive scratching bouts by the hindpaws to the back of the neck, that peaked across 5–15 min post-injection and declined over the ensuing 5–10 min. The number of bouts elicited by PNX-14 in 30 min was dose-dependent across the first three doses examined here. Fig. 5 shows the mean number of bouts elicited by 2, 4 and 8 mg/kg of PNX-14 to be 32 ± 12 (SD) (n=6), 41 ± 21 (n=9) and 75 ± 18 (n=5) per 30 min, respectively. The dose-response curve was curvilinear with the top dose (16 mg/kg) causing only 59 ± 14 (n=4) bouts.

Phoenixin-20 amide and scratching

At 4 mg/kg, PNX-20 evoked 57 ± 29 (n=7) scratching bouts in mice across 30 min in comparison with 7 ± 2 bouts associated with saline (n=6) (Fig. 6).

Nalfurafine and phoenixin-14

Mice were pretreated s.c. at –20 min with either saline or nalfurafine (20 µg/kg). The mean number of scratching bouts subsequently induced by PNX-14 (4 mg/kg) across 30 min was reduced from 46 ± 23 (n=7) in controls to 1 ± 2.6 (n=7) in the nalfurafine group (Fig. 7). Subjectively, all animals pretreated with nalfurafine exhibited no marked motor impairment or sedation.

Discussion

Mass spectrometry identifies a major peak corresponding to the molecular weight of PNX-14 in mouse spinal cord homogenate, whereas the peak corresponding to PNX-20 is not distinct. Though the two small peaks of N-terminal extended peptides PNX-17 and PNX-20 were observed, the fragment of VQEDVQPPGLKVWSDPF-amide (PNX-17) may be postulated as the degraded product of PNX-20.

In the spinal cord, irPNX is detected in a population of small-to-medium sized DRG whose cell processes project to the superficial layers of the dorsal horn and peripherally to target tissues including skin, as illustrated by co-localization of irPNX with the retrograde fluorescent dye Fluorogold in a population of DRG cells. Our behavioral studies show that PNX-14 (2–16 mg/kg) or PNX-20 (4 mg/kg) injected s.c. to the nape of the neck of mice elicited repetitive scratching, which had a latency of 3–5 min, lasted 10–15 min, and was suppressed by nalfurafine, the kappa opioid receptor agonist.

Historically, pruritus has been considered to be a milder form of pain, and as such these two sensory modalities are believed to be closely related insofar as signaling molecule(s) and neural circuitry are concerned (Handwerker, 2014). Recent studies suggest that nociception and pruritus are likely to be mediated by modality-selective signaling molecules or receptors/channels (Bautista et al., 2014; Davidson and Giesler, 2010; Han et al., 2013; Ikoma et al., 2006; Kardon et al., 2014; McNeil et al., 2015; Wilson et al., 2011). In this respect, several peptides including gastrin-releasing peptide (GRP), natriuretic polypeptide B (NPPB), neuromedin B (NMB), and hemokinin-1, have been suggested as endogenous pruritogens (Funahashi et al., 2014; Mishra and Hoon, 2013; Su and Ko, 2011; Sun and Chen, 2007). Utilizing the bioinformatics algorithm from information provided by the Genome Projects, two secreted, highly conserved peptides, PNX-14 and PNX-20, with no known biological functions, were isolated and identified in neural and non-neural tissues (Lyu et al., 2013; Yosten et al., 2013). As mass spectrometry revealed a larger quantity of PNX-14 in the mouse spinal cord as compared with the trace amount of PNX-20, the former was the primary target for this study.

Insofar as the biological role of PNX-14 in sensory processing is concerned, several observations support the contention that the peptide may serve as a signal molecule transducing itch sensation. First, irPNX is expressed in neural elements associated with sensory information processing. Accordingly, irPNX is present in cell processes densely distributed to superficial layers of the dorsal horn, but sparsely to the deeper laminae; and in small-to-medium size DRG cells. This pattern of distribution of irPNX observed here in the mouse spinal cord is comparable to that reported in the rat spinal cord (Lyu et al., 2013). Moreover, irPNX cell processes are detected in the epidermis and dermis. Tract tracing studies showed that some of the DRG, which were irPNX, also expressed the retrograde fluorescent marker, Fluorogold, indicating that a population of DRGs is irPNX and their axons extend to the skin. On the other hand, the observation that not all Fluorogold-containing primary afferent neurons are irPNX, suggests that some of the DRG neurons in question may utilize neuromodulators other than phoenixin, such as GRP, NMB and NPPB (Mishra and Hoon, 2013; Su and Ko, 2011; Sun and Chen, 2007).

The expressional pattern of irPNX cell processes in the spinal cord and skin is similar to that of the itch-provoking peptide, GRP (Sun and Chen, 2007; Tominaga et al., 2009). It is of interest to note that the number of GRP-positive nerve fibers is higher in the skin of NC/Nga mice, an animal model of atopic dermatitis (Tominaga et al., 2009). On the other hand, Goswami et al. (2014) raised a concern that GRP immunoreactivity observed in the dorsal horn may be attributed to cross-reactivity between GRP antiserum and SP or CGRP. To minimize potential cross-reactivity between PNX antiserum and peptides other than

phoenixin, a pre-absorption protocol was implemented in which cross-reactivity of PNX antiserum with SP, GRP, CGRP and PNX-14 was individually assessed. Incubation of spinal sections with PNX antiserum that had been pre-absorbed with the peptide PNX-14 resulted in absence of irPNX in the dorsal horn, whereas irPNX could still be observed in the dorsal horn in spinal sections incubated in PNX antiserum pre-absorbed with SP, CGRP or GRP. Collectively, these results demonstrate that PNX antiserum was selective against phoenixin and exhibited little or no cross-reactivity with the other three peptides.

When injected s.c. to the nape of the neck, PNX-14 consistently evoked scratching behavior characterized by repetitive bouts to the back of the neck with the hindpaw, varying from 16 to 95 bouts in 30 min. Although this behavior was qualitatively similar to that described previously in mice for compound 48/80 (Kuraishi et al., 1995), norbinaltorphimine (norBNI) (Kamei and Nagase, 2001), chloroquine (Inan and Cowan, 2004) and 5'-guanidinonaltrindole (5'-GNTI) (Inan et al., 2011), scratching bouts elicited by PNX-14 occurred less frequently, were less intense, and tapered off sooner than those observed with these known pruritogens. For example, a standard dose of 5'-GNTI (0.30 mg/kg, s.c.) in mice precipitated 579 ± 234 (SD) bouts across 30 min, which tapered off across 30–80 min (Inan et al., 2009). As noted already, the standard dose of PNX-14 (4 mg/kg) caused 41 ± 21 bouts across 30 min with the bouts ceasing after 20 min. PNX-14, being a small peptide, could be rapidly degraded by tissue carboxypeptidase, resulting in fewer bouts and a shorter duration of action relative to the other pruritogens.

It is noteworthy that PNX-14-induced scratching was accompanied by, or followed by, excessive self-grooming of the body, a behavior (Spruijt et al., 1992) that has been reported extensively in mice after central or intra-spinal administration of one of several chemically diverse peptides but much less frequently after peripheral injection. BN (2.7 mg/kg, i.p.) (Cowan et al., 1985) and the xenobiotics 5'-GNTI (0.30 mg/kg, s.c.), norBNI (10 mg/kg, s.c.) and compound 48/80 (2 mg/kg, s.c.) resemble PNX-14 in this respect (unpublished observations). The grooming observed in the present study may be a mechanism by which the mouse's state of arousal, which has been raised by PNX-14-evoked scratching, is lowered and homeostasis is maintained. A recent relevant report by Jiang et al. (2015) ascribes possible anxiolytic activity to PNX-14 when delivered i.c.v. to mice but makes no mention of associated body scratching or grooming by this route of administration.

The site and mechanism whereby phoenixin provokes scratching behavior remains to be further explored. An interesting feature of PNX-induced scratching relates to its antagonism by kappa opioid receptor agonists such as nalfurafine, which has been shown to reduce scratching bouts precipitated by 5'-GNTI (Inan et al., 2009), SP (Togashi et al., 2002), histamine (Togashi et al., 2002), morphine (Wakasa et al., 2004), compound 48/80 (Wang et al., 2005) and chloroquine (Inan and Cowan, 2004). These data imply that kappa opioid receptors are key to the inhibition of scratching by a chemically diverse group of pruritogens. A recent study, however, shows that zyklophin, a short-acting kappa opioid receptor antagonist, induces scratching in wild type as well as in kappa opioid receptor knock out mice (DiMattio et al., 2014). This raises the issue of the obligatory nature of kappa opioid receptors in relation to itch sensation in mice. Kardon et al. (2014) present evidence that dynorphin, the endogenous kappa opioid receptor agonist, acting on kappa

opioid receptors expressed in a selective population of spinal inhibitory neurons, referred to as B5-1 neurons, inhibits dorsal horn glycine/GABA inhibitory neurons, which ultimately contribute to the development of itch sensation by a mechanism of disinhibition of downstream spinal neurons. It may be tempting to speculate that phoenixin, released from primary afferents, excites dynorphin-containing dorsal horn neurons that express kappa opioid receptors, leading to a release of dynorphin, which then inhibits B5-1 neurons, contributing to itch sensation by disinhibition of spinal neurons. What remains to be learned are the signaling molecules and intervening steps between inhibition of B5-1 neurons and spinal output of itch sensation.

Conclusion

The recent description of endogenous pruriceptive peptides such as bovine adrenal medulla 8–22 (Sikand et al., 2011), hemokinin-1, gastrin-releasing peptide and natriuretic peptide B has galvanized the field of itch research and promoted critical discoveries at molecular, receptor, anatomical and behavioral levels that hold promise for developing new antipruritic therapies (Kardon et al., 2014). Our study with phoenixin is expected to prompt similar interest, help to unveil the biological bases of itch, and develop novel, efficacious anti-itch medications.

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Abbreviations

BN	Bombesin
CGRP	calcitonin-gene related peptide
DRG	dorsal root ganglion/ganglia
FITC	fluorescein isothiocyanate
GRP	gastrin-releasing peptide
GRPR	gastrin-releasing peptide receptor
5'-GNTI	5'-guanidinonaltrindole
i.c.v.	intracerebroventricular
NPPB	natriuretic polypeptide B
NMB	neuromedin B
norBNI	norbinaltorphimine
PNX	phoenixin
PNX-14	phoenixin-14 amide
PNX-20	phoenixin-20 amide

irPNX	phoenixin immunoreactivity
PBS	phosphate buffered saline
SP	substance P

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Highlights

- Phoenixin (PNX), a novel peptide of 14 amino acids, is detected in the mouse spinal cord
- PNX is expressed in DRGs with axons projecting to the dorsal horn and peripherally to the skin
- PNX upon subcutaneous injection to the nape of the neck of mice provokes scratching behavior
- PNX-induced scratching is attenuated by nalfurafine, the kappa opioid receptor agonist
- PNX is one of the endogenous signaling molecules involved in itch transmission in the mouse

Data: 05-03-15 mouse spinal crude extract 4a0001
Kratos PC Axima LNR V2.4.1

Fig. 1.

Mass spectrometry analysis of phoenixin in mouse spinal cord homogenate. The major peak corresponds to PNX-14 with a molecular weight of 1583.79. A small peak with a molecular weight of 2182.45 might represent a trace amount of PNX-20.

Fig. 2.

irPNX in mouse spinal cord and dorsal root ganglion. A, a cervical section where irPNX cell processes are detected in the superficial dorsal horn, with few strands of irPNX cell processes extending to deeper laminae. B, a higher magnification of the boxed area from panel A, where irPNX cell processes about on to superficial layers of the dorsal horn. C, a section of dorsal root ganglion where several irPNX cells are indicated by arrows; not all dorsal root ganglion cells are irPNX. D, a section of cervical cord labeled with PNX

antiserum pre-absorbed with the peptide PNx-14 (1 µg/ml); irPNx is not detected in the dorsal horn, as illustrated by the absence of fluorescence.

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Fig. 3. irPNX cell processes in mouse skin. A, several fine, beaded irPNX cell processes, as indicated by arrows, are seen in the dermis. B, a higher magnification of irPNX cell processes, indicated by arrows, with bead-like appearance.

Fig. 4.

A section of dorsal root ganglion labeled with phoenixin antiserum from a mouse injected s.c. with the retrograde tracer Fluorogold four days earlier. A, dorsal root ganglion section where many Fluorogold labeled cells (green color) are detected; some of which are marked by arrowheads. B, same section as A, but labeled with phoenixin antiserum; irPNX cells appear red, and several strongly fluorescent cells are marked by arrows. C, a merged image of A and B, where several dorsal root ganglion cells expressing both Fluorogold and irPNX (orange/yellow color) are indicated by double arrowheads; several Fluorogold filled cells (green color) are not irPNX.

Fig. 5.

Dose-response relationship of PNx-14 in mice. Effect of saline or phoenixin-14 amide (2–16 mg/kg), given s.c. behind the neck, on scratch induction from 0–30 min in mice (n=4–9). (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the saline group; Kruskal-Wallis test then Dunn's multiple comparison test).

Fig. 6. Phoenixin-20-amide induced scratching in mice. Effect of saline (n=6) or phoenixin-20 amide (4 mg/kg; n=7), given s.c. behind the neck, on scratch induction from 0–30 min in mice. (***) $p < 0.001$; Mann-Whitney U test).

Fig. 7.

Attenuation of scratching by nalfurafine. Inhibitory action of nalfurafine (Nal; 0.02 mg/kg, s.c. in the flank at -20 min) on scratching induced from 0-30 min by a submaximal dose of phoenixin-14 amide (4 mg/kg, s.c. behind the neck) in mice (n=7). (***) $p < 0.001$; Mann-Whitney U test). Injections of saline (Sal) and PNX-14 (4 mg/kg) serve as control response.